A bootstrap approach is a superior statistical method for the comparison of cell-to-cell movement data

Matthew G. Johnston¹ and Christine Faulkner

Department of Crop Genetics, John Innes Centre, Norwich, UK

¹Corresponding author: matthew.johnston@jic.ac.uk, +44 (0)1603 450000

Total words: 1176

Total figures: 1

Keywords

Plasmodesmata, bootstrap, Mann-Wilcoxon-Whitney, cell-to-cell movement, statistics

Summary

Plasmodesmata are an increasing focus of plant research, and plant physiologists frequently aim to understand the dynamics of intercellular movement and plasmodesmal function. For this, experiments that measure the spread of GFP between cells are commonly performed to indicate whether plasmodesmata are more open or closed in different conditions or in different genotypes.

We propose cell-to-cell movement data sets are better analysed by a bootstrap method that tests the null hypothesis that means (or medians) are the same between two conditions, instead of the commonly used Mann-Whitney-Wilcoxon test. We found that that with hypothetical distributions similar to cell-to-cell movement data, the Mann-Whitney-Wilcoxon produces a false positive rate of 17% while the bootstrap method maintains a false positive at the set rate of 5% under the same circumstances. Here we present this finding, as well as our rationale, an explanation of the bootstrap method and an R script for easy use. We have further demonstrated its use on published datasets from independent laboratories.

Main Text

Symplastic cell-to-cell connectivity is dynamically regulated in plants as a component of developmental and environmental responses (Perbal *et al.*, 1996; Wada *et al.*, 2002; Faulkner *et al.*, 2013). Connectivity is established between cells by plasmodesmata, which function as a key parameter to define the dynamics of cell-to-cell connectivity. It is critical to assay the degree of movement of different molecules between cells to understand the range and dynamics of cell-to-cell communication as well as to assay plasmodesmal function under different conditions or in different genotypes. Accurate experimental analysis is critical to understanding this important component of plant physiology.

32 There are two routinely used methods, with a cellular resolution, to assay the spread of GREEN
 33 FLUORESCENT PROTEIN (GFP) from one cell into neighbouring cells: microprojectile bombardment,
 34 and low OD₆₀₀ *Agrobacterium tumefaciens* infiltration (Oparka *et al.*, 1999; Burch-Smith & Zambryski,
 35 2010). These assays allow the experimenter to count the number of cells ('cell count'), or the
 36 number of concentric rings of cells ('cell layers'), to which GFP has spread from a single cell (Fig. **1a**).
 37 This serves as a measure of symplastic connectivity – the further the GFP has spread, the greater the
 38 degree of connection (or of plasmodesmata permeability) between cells. Neither cell nor layer
 39 counts are parametrically distributed (Fig. **1b – d**, upper), so most studies use the non-parametric
 40 Mann–Whitney–Wilcoxon (MWW) test to compare conditions to identify factors that regulate the
 41 connection and communication between cells.

42 Most experiments aim to assess whether connectivity is greater or less under different conditions, or
 43 whether plasmodesmata are more open or closed, which involves analysis of changes in the median
 44 or mean. The MWW tests the null hypothesis that two data distributions are the same (Mann &
 45 Whitney, 1947), not whether the two distributions have the same median. Therefore, it is possible to
 46 find a significant difference in an MWW test with distributions that have the same median, but
 47 different variances (Hart, 2001). When data from cell count assays are presented in histogram form,
 48 it is clear that the shape of the distributions differs between experimentally compared conditions or
 49 genotypes (Fig. **1b, c**) (Guseman *et al.*, 2010; Diao *et al.*, 2018; Cheval *et al.*, 2020). Thus, if an MWW
 50 test is used on cell count data, the difference in distribution shapes between conditions may lead to
 51 the erroneous conclusion that there is a significant difference in the amount of spread of GFP.

52 Therefore, a different statistical method is required to properly interpret differences in GFP spread.
 53 For this, we propose a bootstrap method (Efron, 1979). Unlike the MWW test, bootstrapping works
 54 with data that is both non-parametric and heteroskedastic (differing variance between conditions).

55 The goal of the analysis is to estimate the probability that the observed difference in medians ($\hat{\theta}$)
 56 came about by chance (a p value). Frequentist statistics does this by comparing $\hat{\theta}$ to a null
 57 distribution. In this case, the null distribution describes the probability of observing a difference in
 58 medians, when there is no true difference in the underlying data. Usually, a known distribution is
 59 used (e.g. t-distribution or F-distribution) but in this case it is unknown because the data do not
 60 follow parametric distributions (Fig. **1b, c**).

61 Bootstrapping techniques can be used to generate a null distribution *de novo* from the observed
 62 data already collected, as long as the samples are independent. This removes the requirement of
 63 using a known distribution. To do this, the observed data are sampled with replacement to generate
 64 a resample. This mimics what the experimenter has done originally when observing the true

65 population. The relationship between multiple resamples and the observed data can be used to
66 reveal how the observed data relate to the true population, and so estimate a p value for the
67 observation.

68 An example R function is provided to perform this analysis (*medianBootstrap.R*,
69 <https://github.com/faulknerfalcons/Johnston-2020-Bootstrap>), which requires two arguments, i.e.
70 two vectors of numbers: control and treatment. The function generates a null distribution to
71 compare against by resampling each vector N times (by default 5000) and, for each resample,
72 generating a resampled test statistic ($\widehat{\theta}^*$). These N resampled test statistics are made into a null
73 distribution by $|\widehat{\theta}_n^* - \widehat{\theta}|$ (Fig. **1b – c**, lower) as suggested by Hall and Wilson (1991).

74 As this is a random sampling technique, an exact p value cannot be calculated but an estimate is
75 produced: a Monte Carlo $\hat{p}$ value (Eqn. 1). To do so, $\widehat{\theta}$ is compared to the null distribution to find the
76 chance of observing a value at least as extreme (line on Fig. **1b – c**, lower). A +1 is added to the
77 numerator and denominator in Eqn. 1 as suggested by Davison and Hinkley (1997): conceptually, this
78 can be considered as including the observed sample among the bootstrap resamples.

$$\hat{p} = \frac{\sum_{n=1}^N I(|\widehat{\theta}_n^* - \widehat{\theta}| \geq \widehat{\theta}) + 1}{N + 1} \quad \text{Eqn. 1}$$

79 where $I(\cdot)$ is the indicator function.

80 As $\hat{p}$ is an estimate of p , a 95% confidence interval should be constructed, where p will fall within
81 this range 95% of the time (Wilson, 1927).

82 This method is not confounded by differences in variance or shape as with the MWW test. To
83 illustrate this, we compared the Type I error rate (false positives) between the MWW and
84 *medianBootstrap* tests, when testing if there is a difference in medians between two populations for
85 which there was no true difference in medians, i.e. $\theta = 0$. In this scenario, an error rate of 5% is
86 expected at $\alpha = 0.05$. Samples ($n = 100$) for each population were drawn from normal
87 distributions with the same variance ($X, Y \sim N(0, 1)$) simulated in R 4.0.0 (R Core Team, 2020). Both
88 the MWW and *medianBootstrap* methods gave a difference in medians about 5% of the time, as
89 expected (4.5% (95% CI [3.4, 6.0]) and 4.9% (95% CI [3.7, 6.4]), respectively). When variances
90 differed between populations ($X \sim N(0, 1), Y \sim N(0, 5^2)$), the MWW test had a false positive rate
91 significantly higher than the set 5% of 7.5% (95% CI [6.0, 9.3]). Conversely, the false positive rate of
92 the *medianBootstrap* method was correctly controlled at 4.7% (95% CI [3.6, 6.2]).

93 Alternatively, when two samples are drawn from populations with equal variance and median, but
94 differing shape ($X \sim N(1 - \frac{1}{\sqrt[3]{2}}, \frac{3}{80}), Y \sim \text{Beta}(1, 3)$), a *medianBootstrap* method finds a significant

difference in 5.1% of the trials (95% CI [3.9, 6.6]), as expected. Whereas, an MWW test inflates the Type I error rate to 17% (95% CI [15, 19]). Therefore, as cell count data exhibit unequal variances and differing distribution shapes between conditions and/or genotypes, we propose that bootstrap methods are a more appropriate analysis to identify differences in the spread of GFP. It is worth noting that any test statistic, θ , can be computed in a bootstrapped manner, provided the test is invariant to scaling. This means bootstrap testing can be extended to cell layer data, where means should be compared, as there is no difference in medians (Fig. 1d). An example of this extension is given in *medianBootstrap.html*.

We acknowledge alternative advanced statistical techniques, such as linear mixed effects models, for the analysis of these data. However, they require more assumptions and are less user-friendly. We consider this bootstrap method a good, easy-to-use, superior alternative to MWW analysis of cell-to-cell movement data.

Acknowledgments

We thank Joanna Jennings (Department of Crop Genetics, John Innes Centre) for providing the confocal micrograph in Fig. 1a and Dr Joshua Hodgson (Department of Medicine, University of Cambridge) and Dr Matthew Castle (Department of Genetics, University of Cambridge) for constructive comments on the manuscript. The data in Fig. 1 comes from (b) Figure S2 of Cheval *et al.* (2020), (c) Figure 2d Diao *et al.* (2018), (d) Figure 2c Diao *et al.* (2018) under use of the CC BY 4.0 licence. MGJ is funded by a John Innes Foundation Studentship. Research in the Faulkner lab is supported by the Biotechnology and Biological Research Council Grant (BB/L000466/1, BBS/E/J/000PR9796) and the European Research Council (725459, “INTERCELLAR”).

Author Contributions

MGJ and CF designed, discussed and wrote up the research. MGJ performed the analysis.

References

- Burch-Smith TM, Zambryski PC. 2010. Loss of increased size exclusion limit (ise)1 or ise2 increases the formation of secondary plasmodesmata. *Current Biology* **20**: 989–993.
- Cheval C, Samwald S, Johnston MG, de Keijzer J, Breakspear A, Liu X, Bellandi A, Kadota Y, Zipfel C, Faulkner C. 2020. Chitin perception in plasmodesmata characterizes submembrane immune-signaling specificity in plants. *Proceedings of the National Academy of Sciences* **117**: 9621–9629.
- Davison AC, Hinkley D V. 1997. *Bootstrap Methods and their Application*. Cambridge University Press.
- Diao M, Ren S, Wang Q, Qian L, Shen J, Liu Y, Huang S. 2018. Arabidopsis formin 2 regulates cell-to-cell trafficking by capping and stabilizing actin filaments at plasmodesmata. *eLife* **7**: e36316.
- Efron B. 1979. Bootstrap Methods: Another Look at the Jackknife. *The Annals of Statistics* **7**: 1–26.

- Faulkner C, Petutschnig E, Benitez-Alfonso Y, Beck M, Robatzek S, Lipka V, Maule AJ. 2013.** LYM2-dependent chitin perception limits molecular flux via plasmodesmata. *Proceedings of the National Academy of Sciences* **110**: 9166–9170.
- Guseman JM, Lee JS, Bogenschutz NL, Peterson KM, Virata RE, Xie B, Kanaoka MM, Hong Z, Torii KU. 2010.** Dysregulation of cell-to-cell connectivity and stomatal patterning by loss-of-function mutation in Arabidopsis CHORUS (GLUCAN SYNTHASE-LIKE 8). *Development* **137**: 1731–1741.
- Hall P, Wilson SR. 1991.** Two Guidelines for Bootstrap Hypothesis Testing. *Biometrics* **47**: 757–762.
- Hart A. 2001.** Mann-Whitney test is not just a test of medians: Differences in spread can be important. *British Medical Journal* **323**: 391–393.
- Mann HB, Whitney DR. 1947.** On a Test of Whether one of Two Random Variables is Stochastically Larger than the Other. *The Annals of Mathematical Statistics* **18**: 50–60.
- Oparka KJ, Roberts AG, Boevink P, Cruz SS, Roberts I, Pradel KS, Imlau A, Kotlizky G, Sauer N, Epel B. 1999.** Simple, but not branched, plasmodesmata allow the nonspecific trafficking of proteins in developing tobacco leaves. *Cell* **97**: 743–754.
- Perbal MC, Haughn G, Saedler H, Schwarz-Sommer Z. 1996.** Non-cell-autonomous function of the Antirrhinum floral homeotic proteins DEFICIENS and GLOBOSA is exerted by their polar cell-to-cell trafficking. *Development* **122**: 3433–41.
- R Core Team. 2020.** R: A Language and Environment for Statistical Computing.
- Wada T, Kurata T, Tominaga R, Koshino-Kimura Y, Tachibana T, Goto K, Marks MD, Shimura Y, Okada K. 2002.** Role of a positive regulator of root hair development, CAPRICE, in Arabidopsis root epidermal cell differentiation. *Development* **129**: 5409–19.
- Wilson EB. 1927.** Probable Inference, the Law of Succession, and Statistical Inference. *Journal of the American Statistical Association* **22**: 209–212.

Figure 1 Bootstrap statistics on GFP movement data.

(a) An example image of GFP moving from a single transformation site. The degree of movement can either be counted as the number of fluorescent cells (denoted with stars, 17 cells) or the number of cell layers with GFP (blue overlays, 3 layers). Scale bar = 100 μ m. **(b – d)** *Top*: Histogram of cell counts or layers, with the median and mean marked. *Bottom*: Bootstrap null distributions ($|\hat{\theta}^* - \hat{\theta}|$) for the differences in **(b, c)** median or **(d)** mean, with estimated $\hat{p}$ value and 95% confidence intervals (CI). The observed difference ($\hat{\theta}$) is marked by a red line. Data for **(b)** from Cheval *et al.* (2020) and data for **(c, d)** from Diao *et al.* (2018) both under the CC BY 4.0 licence.

a)

- Median
- Mean
- θ Test statistic
- $\hat{\theta}$ Sampled test statistic
- $\hat{\theta}^*$ Resampled test statistic
- Δ_μ Difference in means
- Δ_{median} Difference in medians

b)

c)

d)